RELATIVE CONTRIBUTIONS OF THE FRACTION OF UNFROZEN WATER AND OF SALT CONCENTRATION TO THE SURVIVAL OF SLOWLY FROZEN HUMAN ERYTHROCYTES

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ABSTRACT As suspensions of cells freeze, the electrolytes and other solutes in the external solution concentrate progressively, and the cells undergo osmotic dehydration if cooling is slow. The progressive concentration of solute comes about as increasing amounts of pure ice precipitate out of solution and cause the liquid-filled channels in which the cells are sequestered to dwindle in size. The consensus has been that slow freezing injury is related to the composition of the solution in these channels and not to the amount of residual liquid. The purpose of the research reported here was to test this assumption on human erythrocytes. Ordinarily, solute concentration and the amount of liquid in the unfrozen channels are inversely coupled. To vary them independently, one must vary the initial solute concentration. Two solutes were used here: NaCl and the permeating protective additive glycerol. To vary the total initial solute concentration while holding the mass ratio of glycerol to NaCl constant, we had to allow the NaCl tonicity to depart from isotonic. Specifically, human red cells were suspended in solutions with weight ratios of glycerol to NaCl of either 5.42 or 11.26, where the concentrations of NaCl were 0.6, 0.75, 1.0, 2.0, 3.0, or 4.0 times isotonic. Samples were then frozen to various subzero temperatures, which were chosen to produce various molalities of NaCl (0.24–3.30) while holding the fraction of unfrozen water constant, or conversely to produce various unfrozen fractions (0.03–0.5)while holding the molality of salt constant. (Not all combinations of these values were possible). The following general findings emerged: (*a*) few cells survived the freezing of > 90% of the extracellular water regardless of the salt concentration in the residual unfrozen portion. (*b*) When the fraction of frozen water was < 75%, the majority of the cells survived even when the salt concentration in the unfrozen portion exceeded 2 molal. (*c*) Salt concentration affected survival significantly only when the frozen fraction lay between 75 and 90%. To find a major effect on survival of the fraction of water that remains unfrozen was unexpected. It may require major modifications in how cryobiologists view solution-effect injury and its prevention.

INTRODUCTION

It has become clear that freezing injury is a consequence of at least two categories of physicochemical events. One, associated with rapid cooling, is the formation of intracellular ice crystals and their subsequent growth during slow warming. The other, associated with slow cooling, is a consequence of the changes in the extra- and intracellular solution brought about

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by the conversion of water into ice. Intracellular freezing is usually lethal regardless of whether protective solutes like glycerol are present. But the lethality of the second category, solution effects (Mazur, 1970), can be reduced or even eliminated by the presence of protective solutes in suitable concentrations.

As aqueous solutions freeze, the cells and the solutes in the external solution become progressively concentrated in unfrozen aqueous channels between the ice crystals. In response to the increased external solute concentration, slowly frozen cells shrink osmotically. These two phenomena, solute concentration and cell shrinkage, have formed the basis of the two most widely held theories of slow-freezing injury. The first, originally proposed quantitatively by Lovelock (1953*a,b*), is that slow freezing injury occurs when extra- and intracellular electrolytes reach critical concentrations during freezing. According to this hypothesis, the protective effect of permeating solutes like glycerol and dimethyl sulfoxide results from their colligative action to reduce electrolyte concentration at a given subzero temperature. (We discuss the quantitative aspects of this colligative effect later in this paper.)

The alternative hypothesis, that freezing injury is a consequence of the shrinkage of cells as they respond osmotically to increased external concentrations, was first proposed by Meryman (1968, 1974). To be more precise, the hypothesis states that cells tend to resist shrinkage below a certain critical volume. This results in osmotic disequilibrium and leads to membrane injury. According to this critical volume hypothesis, the protective effect of solutes like glycerol and dimethyl sulfoxide results from their colligative action to reduce the extent of cell shrinkage at any temperature.

A variant of this second hypothesis has been recently proposed by Steponkus and his colleagues (see Steponkus and Wiest, 1978). According to their view, cells that have shrunk osmotically during freezing can tolerate only a certain increase in surface area during thawing. Damage results if that critical surface area increment is exceeded.

The two (or three) hypotheses agree on one point: injury during slow freezing is either a direct or secondary consequence of the lowered chemical potential of water in the external medium, a lowering brought about by the increased solute concentration.

There is, however, a third phenomenon that occurs during the freezing of aqueous solutions: the progressive increase in solute concentration with the lowering of temperature comes about from a progressive reduction in the fraction of the solution that remains unfrozen. This means that during freezing the volume of liquid in the channels in which the cells are sequestered decreases as water is converted into ice. Most investigators (including us) have heretofore dismissed the idea that the amount of unfrozen liquid, as opposed to its composition, could influence cell survival. There was no logical reason to favor such a view, and therefore little thought had been given as to how to separate the two effects experimentally. Experiments, for example, designed to test the effect of lowering the extracellular salt concentration during freezing by raising the glycerol concentration will simultaneously increase the fraction of the solution that remains unfrozen at a given temperature (see Rall et al., 1978).

There is, however, a straightforward way to vary solute concentration and the unfrozen fraction independently. The way of doing so and the results of doing so are the subject of this paper. The results proved unexpected, and even astonishing, for they show that the survival of the slowly frozen human erythrocyte is far more dependent on the fraction of water that remains unfrozen than it is on the concentration of salt (NaCl) in that unfrozen water.

MATERIALS AND METHODS

Human blood cells collected in heparinized tubes were washed twice by centrifugation with a solution of 0.15 M NaCl (8.68 g/liter). The washed cells were then suspended in 0.15 M NaCl at the same hematocrit as in the original whole blood. The collected blood, stored at 4°C, was used for no longer than 5 d. Washed cells were used the same day.

Preparation of Suspensions of Cells in Solutions of Glycerol in Isotonic NaCl

A 0.25-ml portion of washed cells was mixed with 4.75 ml of isotonic NaCl (8.57 g/kg H_2O) containing 0, 0.5, 1.0, 1.5, or 2.0 M glycerol. Because of the slight dilution by the saline transferred with the cells, the molar concentrations of glycerol in the resulting cell suspensions were 97% of the above values, which we shall refer to as nominal. The exact compositions of the resulting solutions are identical to those of Rall et al. (1978, Table IA).

Preparation of Suspensions of Cells in Glycerol-NaCl-water Solutions Designed to Separate the Effects of NaCl Concentration from the Effects of the Fraction of the Water that is Frozen

Cell suspensions were prepared by adding 4.75 ml of appropriate glycerol-NaCl-water solutions in seven steps to a 0.25-ml vol of washed erythrocytes. 12 different solutions of glycerol-NaCl-water were used. Six were of such composition that when the washed cells were added to them they contained 0.6, 0.75,

TABLE I
COMPOSITION OF EXPERIMENTAL GLYCEROL-NaCl-WATER SOLUTIONS

Solution*	Percent by weight‡			Molality		
	NaCl	Glycerol	Water	NaCl	Glycerol	V_c§
	W_s°	W_g°	W_w°	m_s°	m_g°	
R = 5.42‖						
R5-0.6X-G3	0.520	2.819	96.66	0.092	0.317	1.44
R5-0.75X-G3	0.610	3.308	96.08	0.109	0.374	1.27
R5-1X-G4	0.813	4.410	94.78	0.147	0.505	1.03
R5-2X-G9	1.626	8.819	89.55	0.311	1.069	0.68
R5-3X-G13	2.439	13.23	84.33	0.495	1.703	0.58
R5-4X-G18	3.252	17.64	79.11	0.703	2.421	0.56
R = 11.26						
R11-0.6X-G6	0.504	5.674	93.82	0.092	0.657	1.46
R11-0.75X-G7	0.582	6.552	92.87	0.107	0.766	1.31
R11-1X-G9	0.776	8.735	90.49	0.147	1.048	1.05
R11-2X-G18	1.552	17.47	80.98	0.328	2.343	0.72
R11-3X-G26	2.328	26.21	71.47	0.557	3.982	0.68
R11-4X-G35	3.104	34.94	61.95	0.857	6.124	0.72

*In the descriptor "R5-0.6X-G3," for example, "R5" refers to the R value, "0.6X" is the percent by weight NaCl relative to the percent by weight in the isotonic solution, and "G3" represents approximate percent by weight of glycerol.

‡Composition of the solution after 1 vol of washed erythrocytes in isotonic saline was mixed with 20 vol of stock solutions of glycerol-NaCl-water.

§Computed volume of red cells suspended in these solutions relative to their volume in 0.147 molal (isotonic) NaCl. Calculated by Eq. 6. In the R11-4X-G35 solution the volume of intracellular glycerol more than compensates for the loss of water.

‖$R = W_g^\circ / W_s^\circ$.

1.0, 2.0, 3.0, and 4.0 times the isotonic concentrations (percent by weight) of NaCl, and they contained correspondingly increasing concentrations of glycerol so that the weight ratio of glycerol to NaCl (R) remained at 5.42 in all six solutions. The second set of solutions also contained relative NaCl concentrations of 0.6, 0.75, 1.0, 2.0, 3.0, and 4.0 times isotonic, but the glycerol was present in sufficient concentrations so that R was 11.26. The significance of these weight ratios will be discussed shortly.

The exact percentages by weight of the 12 experimental solutions are given in Table I. To achieve precision the stock solutions to which the washed cells were added to yield the experimental solutions of Table I were prepared gravimetrically. Table I also gives the molalities of glycerol and NaCl in the experimental solutions, and the computed volumes of the erythrocytes suspended in them.

Equilibration with Glycerol

The cell suspensions (now 1/20 of the cell concentration in whole blood) were held at room temperature (22°C) for 30–40 min to allow full permeation of the glycerol (Mazur and Miller, 1976). During this interval, 0.2-ml samples were distributed to 7 × 90-mm glass tubes and mounted on Lucite holders.

Freezing

After equilibration with glycerol, the sample tubes were placed in a bath containing ethanol 2–3°C below the freezing point of the suspending solutions. About 3 min later the supercooled suspensions were made to freeze by tilting the tubes and touching the outside of the tube with a metal rod previously chilled in liquid nitrogen at the point where the slanted surface of the sample meniscus contacted the tube wall.

Seeded samples were held an additional 5 min to allow equilibration with respect to crystallization. The temperature of the ethanol in the freezing bath (FTS Systems Inc., Stone Ridge, N.Y.) was then allowed to fall at either 1.8 (1.79 ± 0.04) °C/min (slow cool) or 0.6 (0.57 ± 0.004) °C/min (very slow cool).

The ethanol bath temperatures were read to ±0.01°C with a Hewlett-Packard quartz thermometer (Hewlett-Packard Co., Palo Alto, Calif.) calibrated with an ice bath. Tests with thermocouples in parallel tubes containing the test solution showed that the temperatures of the samples agreed with the bath temperature within 0.2°C. Triplicate sample tubes were removed from the bath when its temperature fell to the desired temperature (+0, −0.02°C), and were immediately thawed.

Warming and Thawing

Samples were thawed rapidly by shaking the tubes in a 35°C water bath. Immediately after ice disappeared (<30 s), the sample tubes were plunged into an ice bath. The mean warming rate, based on the time for the samples to pass from −65 to −10°C, was ~550°C/min.

Dilution and Determination of Hemolysis

Thawed samples were kept in an ice bath until the samples subjected to the lowest temperatures had been thawed. The 0.2 ml in the thawed suspensions was carefully transferred by Pasteur pipette into the bottom of a centrifuge tube that contained 1.80 ml of the original suspending medium. The freezing tube was back-flushed twice with aliquots of the clear upper portion of the 2 ml of medium, and the contents of the centrifuge tube were then mixed. After centrifugation at 8,000 g for 10 min, 1.0 ml of the supernatant solution of diluted samples was carefully removed by pipette and mixed with 1.0 ml of Drabkin's solution (Wintrobe, 1967). After 10–60 min, the absorbance (A) of cyanmethemoglobin was read at 540 nm in a Bausch & Lomb Spectronic 20 colorimeter (Bausch & Lomb, Inc., Rochester, N.Y.). The absorbance value for 100% hemolysis was obtained by diluting 0.02 ml of washed cells with 3.98 ml of distilled water. Percent hemolysis was calculated as 100 $A_{exp}/A_{100\%}$. Percent survival is the percentage of unhemolyzed cells. Further details on these procedures have been given elsewhere (Miller and Mazur, 1976).

It should be noted that the percent survivals after treatment are for cells still suspended in the original glycerol-NaCl-water solution.

RESULTS

Survival of Human Erythrocytes vs. the Phase Compositions of Partly Frozen Solutions of Glycerol-NaCl-Water

The protective solutes in cryobiological solutions are ordinarily added to isotonic saline. The concentrations of additive and salt that occur during freezing can be obtained from ternary phase diagrams. Of specific concern in this first section are the phase compositions during freezing of solutions of glycerol in 0.85% (wt/vol) (isotonic) NaCl. The phase diagram, a plot of melting point vs. percent by weight of glycerol plus NaCl, is shown in Fig. 1 for two weight percent ratios of glycerol-to-salt (R) in the initial solution (5.42 and 11.26). These curves, or isopleths, were calculated from published data of Goldston (1974) and Shepard et al. (1976) by the methods given by Rall et al. (1978). The R value of 5.42 in the initial unfrozen solution corresponds to 0.5 M glycerol in 0.15 M NaCl. The R value of 11.26 corresponds to 1.0 M glycerol in 0.15 M NaCl.

During freezing, pure water is removed from the solution as ice, and this causes the residual

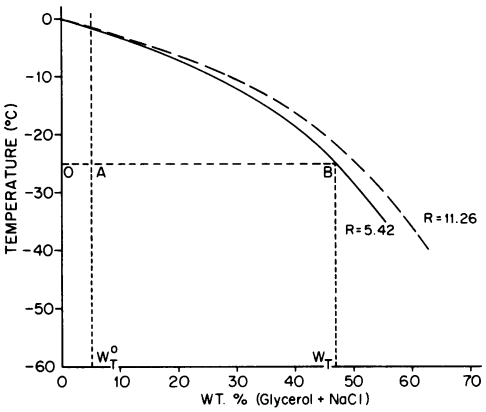

FIGURE 1

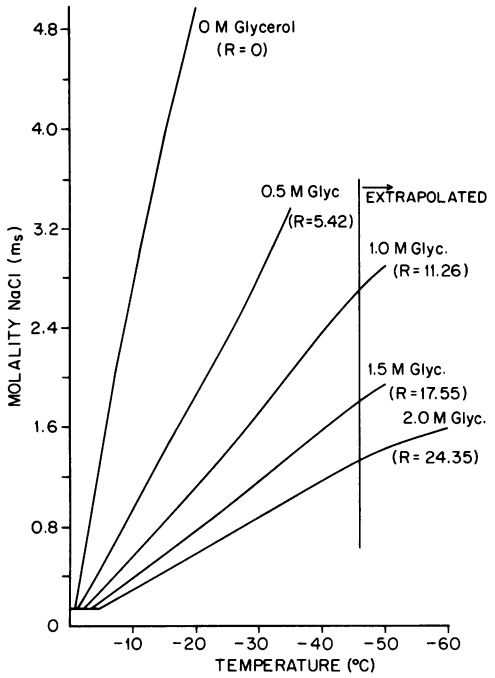

FIGURE 2

FIGURE 1 Equilibrium phase diagrams for the ternary system glycerol-NaCl-water for solutions with weight percent ratios of glycerol to NaCl of 5.42 and 11.26. The curves are interpolated from the data of Goldston (1974) and Shepard et al. (1976). The lines OAB, AW_T^o, and BW_T are discussed in the text.

FIGURE 2 Molality of NaCl(m_s) in the unfrozen portions of glycerol-NaCl-water solutions at various subzero temperatures. The indicated nominal molarities of glycerol (see Materials and Methods) apply only to solutions in which the initial concentration of NaCl is isotonic. However, the curves themselves apply to any glycerol-NaCl solution with the stated R value.

unfrozen solution to concentrate in the manner shown by the isopleths. The R value thus remains unchanged above the line of twofold saturation. The concentration of solute (glycerol + NaCl) at various subzero temperatures can be obtained by drawing lines such as OAB and BW_T to the isopleth. At -25°C, for example, the percent by weight of glycerol plus NaCl in the still unfrozen portion of an $R = 5.42$ solution is 46.6%.

From the total solute concentration and the R value, one can compute the individual weight percents of glycerol and NaCl at various temperatures, and by appropriate formulae convert them to more useful units of concentration (Rall et al., 1978). Thus, Fig. 2 shows the molality of NaCl (m_s) vs. temperature in the unfrozen portion of solutions which initially contained 0, 0.5, 1.0, 1.5, or 2.0 M glycerol in 0.147 molal NaCl. This figure illustrates a point considered important to cryobiology, namely, that the presence of glycerol (or any highly soluble solute) in molar concentrations substantially reduces the concentration of NaCl in the residual unfrozen solution at a given subzero temperature.

Rall et al. (1978) presented data on the survival of human erythrocytes after freezing to various subzero temperatures in glycerol-NaCl solutions of the compositions just noted. We can, therefore, combine their data with those in Fig. 2 to generate a plot of the survival of erythrocytes vs. the molal concentration of NaCl produced during freezing (Fig. 3). The NaCl concentration has a major effect. For example, in 0.5 and 1.0 M glycerol in isotonic saline, survivals are > 80% when the salt concentration remains < 1.0 molal during freezing, but they drop to 20% or less when it rises to 2.0 molal or more. This apparent major dependency of survival on NaCl concentration was first quantitatively demonstrated by Lovelock (1953*a*),

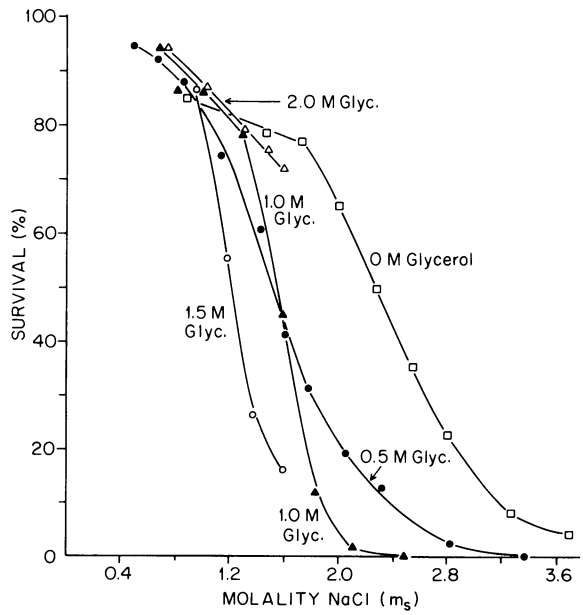

FIGURE 3 Survival (percent unhemolyzed cells) of human erythrocytes as a function of the molality of NaCl(m_s) to which they are exposed after being frozen at 1.8°C/min to various subzero temperatures while suspended in solutions of the indicated molarity of glycerol in isotonic NaCl.

and it is the basis of the theory that the concentration of electrolytes by ice formation is the major factor in slow freezing injury.

But this interpretation is not unique. Solutes concentrate during freezing because of the progressive reduction in the fraction of the solution remaining unfrozen. This unfrozen fraction of the solution can also be obtained from the phase diagram data in Fig. 1. It is given by the ratio OA/OB, which is equivalent to the starting solute concentration W_T^o divided by the solute concentration at the given subzero temperature (W_T).

In Fig. 2 we expressed solute concentration as molalities, i.e., moles/kilogram (≈moles/liter) of water. The counterpart of expressing concentrations in terms of water is to express the unfrozen fractions as the fraction of water remaining unfrozen (U) at various temperatures. These latter data are plotted in Fig. 4 for the same series of glycerol solutions as in Fig. 2. One sees that the higher the concentration of glycerol, the greater is U at any temperature.

These data on fractions unfrozen were then combined with the survival data of Rall et al. (1978) to obtain the plot shown in Fig. 5. In the case of solutions of 0.5 and 1 M glycerol in isotonic NaCl, survivals are > 80% when the fraction unfrozen is 0.14 or more, and they are < 20% when the fraction unfrozen is 0.07 or less.

The question is: is the hemolysis of red cells during slow freezing the result of the attainment of NaCl concentrations >1 molal, as indicated by the curves in Fig. 3, or is it a

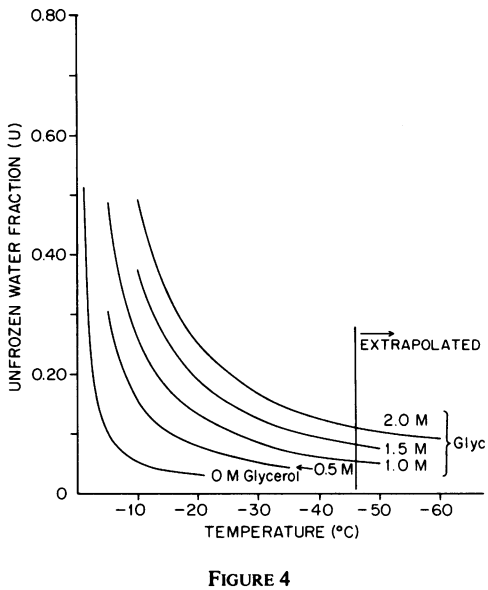

FIGURE 4

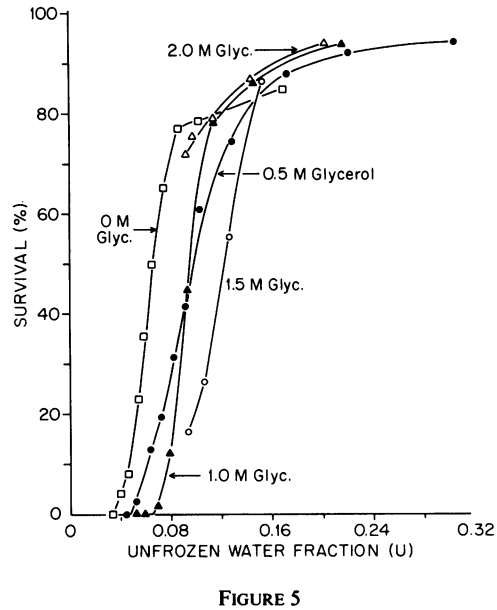

FIGURE 5

FIGURE 4 The fraction of water (U) in glycerol-NaCl-water solutions remaining unfrozen at various subzero temperatures. Unlike the curves in Fig. 2, these curves apply only to solutions which contain the specified molarities of glycerol in isotonic NaCl.

FIGURE 5 Survival (percent unhemolyzed cells) of human erythrocytes as a function of U after the cells have been frozen at 1.8°C/min to various subzero temperatures while suspended in solutions of the indicated molarity of glycerol in isotonic saline.

result of the reduction of the fraction of unfrozen water to below 0.10, as indicated by the curves in Fig. 5?

It will be noted that the curves of survival vs. m_s in Fig. 3 shift to the left with increasing concentrations of glycerol, and that the curves of survival vs. the U in Fig. 5 shift to the right with increasing concentration of glycerol. In other words, as the concentration of glycerol was raised, the cells succumbed at a lower molality of NaCl or when a smaller fraction of extracellular water was frozen. As discussed by Rall et al. (1978), this shift seems to reflect osmotic damage from the glycerol itself.

An alternative to expressing salt concentrations and unfrozen fractions in terms of the amount of liquid *water* is to express them in terms of the amount of liquid *solution.* But when this is done by plotting survival vs. percent by weight of NaCl or vs. the mass fraction of unfrozen solution, the shift in the position of the curves with glycerol concentration becomes much more pronounced than in Figs. 3 and 5 (see Rall et al. [1978] for the necessary equations). In Fig. 5 the median lethal value of U shifts from 0.065 in 0 M glycerol to 0.123 in 1.5 M glycerol. In plots of survival vs. the mass fraction of unfrozen solution, however, the median lethal value of the unfrozen fraction (L) shifts from 0.072 in 0 M glycerol to 0.242 in 1.5 M glycerol. For this reason, when we turned our attention to separating the effects of NaCl concentration from the effects of the unfrozen fraction, we decided to base the separation on the molal concentration of NaCl (m_s) and the mass fraction of unfrozen water, (U), the two measures that showed the smaller influence of glycerol concentration.

Separating the Effects of m_s from Those of U

Inspection of Fig. 1 shows that the solute concentration at a given subzero temperature (W_T) and the unfrozen fraction (W_T^o/W_T) cannot be varied independently as long as the initial solute concentration W_T^o is fixed. The initial solute concentration can be expressed as the concentration of NaCl + (NaCl × R). Consequently, as long as the NaCl concentration is kept isotonic, then both the fraction unfrozen at a given subzero temperature and the concentration of NaCl in that unfrozen fraction will be fixed in a solution with a given R value. Conversely, the only way to vary the NaCl concentration independently from the fraction unfrozen is to vary the starting concentration of glycerol + NaCl (W_T) while holding R, the ratio of the two, constant. To do this, one must suspend the cells in solutions containing NaCl concentrations that are above or below isotonic. For example, a solution of 0.5 M glycerol in isotonic (0.147 molal) NaCl and one of 2.0 M glycerol in 0.3 molal NaCl will both have R = 5.4, but W_T^o will differ by a factor of 2 (Table I). Exactly this approach was used in the present experiments. We restricted the studies to solutions with the two R values shown in Fig. 1, namely, 5.42 and 11.26.

There are limits to which the NaCl concentration and fraction unfrozen can be varied independently because there are limits to which erythrocytes can withstand NaCl concentrations above or below isotonic. The upper limit for NaCl concentration is about seven times isotonic. Studies by Zade-Oppen (1968) and others have shown that exposure to higher concentrations itself produces increasing hemolysis with increasing rapidity.

The lower limit for NaCl concentration is set by the maximum tolerable volume of the erythrocyte (osmotic fragility). Because NaCl is the only species in solutions of glycerol-NaCl-water that cannot permeate erythrocytes, its osmolal concentration alone will determine

the equilibrium volume of water in the erythrocyte, i.e., the volume of water it possesses when the external glycerol has fully permeated the cells. The volume of the whole cell is principally determined by the volume of its water, but there is also a small contribution from the volume of the intracellular glycerol. For example, as shown in Table I, the relative cell volume in the R-11-1X solution is 105% of isotonic. At NaCl concentrations below isotonic, the cells swell. But in erythrocytes there is a sharp limit to the degree of swelling tolerated. In human erythrocytes, for example, ~50% hemolyze when the volume reaches 1.8 times isotonic. Our experimental data on the fragility of human erythrocytes (e.g., Mazur and Miller, 1976) show that at room temperature > 90% will survive (i.e., remain unhemolyzed) in 0.09 molal NaCl, which is 60% of isotonic (here defined as 0.147 molal NaCl). Accordingly, we selected 0.6 times isotonic as the lower limit. We found, however, that cells suspended in solutions of glycerol with that concentration of NaCl underwent ~25% hemolysis after they were chilled to −3 to −5°C. We therefore thought it desirable to introduce an NaCl concentration of 0.75 times isotonic into the series because in that medium hemolysis was only ~10% after the cells were chilled.

In summary then, the experiments were performed using solutions of glycerol-NaCl-water in which the weight ratios of glycerol to NaCl were 5.42 and 11.26 and in which the NaCl concentrations were 0.6, 0.75, 1.0, 2.0, 3.0, or 4.0 times isotonic. The precise compositions of the initial unfrozen solutions are given in Table I.

As noted, the concentration of salt achieved during freezing depends only on the R value of the solution and the temperature, and is not affected by the starting concentration. Consequently, in Fig. 2, which plots m_s vs. temperature, the curve labeled $R = 5.42$ is applicable to all six of the $R = 5.42$ solutions in Table I, and the curve labeled $R = 11.26$ is applicable to all six of the $R = 11.26$ solutions.

This is not the case with the plots of the fraction of unfrozen water vs. temperature in Fig. 4.

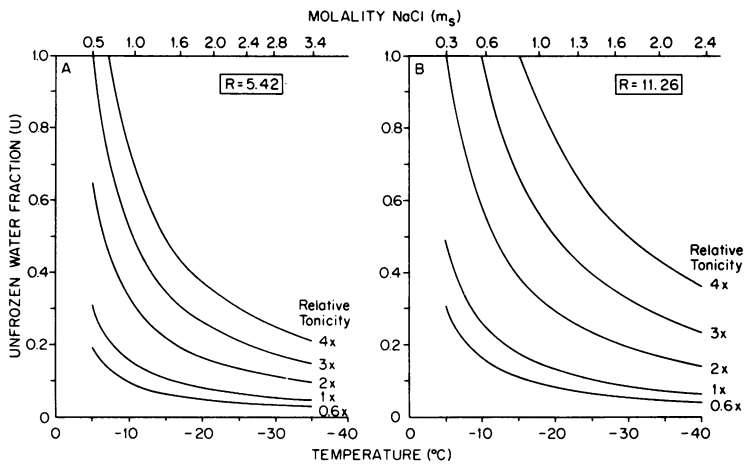

FIGURE 6 U vs. temperature for glycerol-NaCl-water solutions: (A) $R = 5.42$; (B) $R = 11.26$. In both A and B the initial concentration of NaCl ranges from 0.6 to 4 times isotonic. The compositions of the solutions before freezing are given in Table I. The upper abscissas show the molality of NaCl (m_s) that is present in the unfrozen portions of the solution at the indicated temperatures.

As discussed in connection with Fig. 1, the fraction unfrozen (U) depends on the starting concentration as well as on R and temperature. Plots of U vs. temperature for 10 of the 12 solutions in Table I are given in Fig. 6*A* and *B*. This figure also includes on the upper abscissa the values of m_s that correspond to the temperatures.

A vertical line drawn in Fig. 6 yields sets of conditions in which U varies while m_s, is held constant. For example, if the five solutions in Fig. 6 *A* are frozen to -10.7°C, m_s will be 1.0 molal, but U wil range from 0.1 to 0.70.

A horizontal line in Fig. 6 provides sets of conditions in which m_s varies while U is held constant. Thus, if 0.6, 1.0, 2.0, 3.0, and 4.0 times isotonic solutions are frozen to -5, -7.5, -16, -26, and -36°C, respectively, all will have $U = 0.2$, but m_s will range from 0.5 to 3.4 molal.

The curves in Fig. 6 and the temperatures required to achieve desired values of m_s and U in the several solutions were calculated as follows.

1. Temperatures corresponding to m_s values of 1.0, 1.6, 2.0, 2.4, 2.8, and 3.2 were read from the $R = 5.42$ and 11.26 curves in Fig. 2.

2. The total weight of glycerol + NaCl (W_T) at those temperatures was calculated according to the equation

$$W_T = 5844\, m_s/[1{,}000/(R + 1) + 58.44\, m_s] \quad (1)$$

derived from Eqs. 3 and 5 of Rall et al. (1978).

3. The values of U corresponding to each value of m_s were calculated from the equation

$$U = W_T^\circ(100 - W_T)/W_T(100 - W_T^\circ), \quad (2)$$

where W_T° is the total percent by weight glycerol plus NaCl in the initial solution given in Table 1 (see Rall et al., 1978, Eqs. 4 and 7).

4. We then calculated for each of the solutions the m_s values corresponding to U values of 0.075, 0.15, 0.30, 0.45, 0.60, and 0.75. To do this W_T was first calculated by rearranging Eq. 2:

$$W_T = 100\, W_T^\circ/[U(100 - W_T^\circ) + W_T^\circ]. \quad (3)$$

Then the value of m_s corresponding to that value of W_T was calculated by a rearrangement of Eq. (1):

$$m_s = 1{,}000\, W_T/[58.44(R + 1)\,(100 - W_T)]. \quad (4)$$

5. Finally, the temperatures corresponding to the values of m_s were read from the plots in Fig. 2.

Relative Contributions of m_s and U to the Survival of Frozen-Thawed Human Erythrocytes

COOLING AT 1.8°C/MIN, RAPID THAW

Survival vs. temperature. Suspensions of cells in each of the 12 glycerol-NaCl-water solutions were frozen at 1.8°C/min to about eight different temperatures and then thawed rapidly. The temperatures, the corresponding values of m_s and U, the number of replicate samples, and the resulting survivals are given in Table II. The column labeled "absolute

TABLE IIA

SURVIVALS OF HUMAN ERYTHROCYTES SUSPENDED IN VARIOUS GLYCEROL-NaCl-WATER SOLUTIONS, FROZEN AT 1.8°C/MIN TO VARIOUS SUBZERO TEMPERATURES, AND THAWED RAPIDLY

Condition	R	Relative NaCl tonicity	Temperature	Molal. NaCl	Fraction unfrozen	N	Absol. survl.		Norm. survl.	
							Mean	SE	Mean	SE
			(°C)	(m_s)	(U)		(%)		(%)	
R5-0.6X-G3*										
1	5.4	0.6	−3.1	0.31	0.300	6	73.9	5.4	100.0	7.3
2	5.4	0.6	−6.4	0.61	0.150	6	68.1	2.2	92.1	3.0
3	5.4	0.6	−10.7	1.00	0.092	6	17.7	2.2	23.9	3.0
4	5.4	0.6	−13.2	1.23	0.075	6	7.2	2.2	9.8	3.0
5	5.4	0.6	−16.8	1.60	0.058	6	6.7	2.3	9.1	3.1
6	5.4	0.6	−21.5	2.00	0.046	9	10.8	2.6	14.6	3.5
7	5.4	0.6	−26.0	2.40	0.038	9	4.7	1.7	6.3	2.2
8	5.4	0.6	−29.8	2.80	0.033	6	7.1	2.1	9.5	2.8
R11-0.6X-G6										
9	11.3	0.6	−5.2	0.31	0.300	6	73.6	6.4	100.0	8.7
10	11.3	0.6	−10.8	0.61	0.150	6	60.9	7.4	82.9	10.1
11	11.3	0.6	−17.8	1.00	0.092	3	15.3	0.8	20.7	1.2
12	11.3	0.6	−21.9	1.23	0.075	6	14.7	2.1	20.0	2.8
13	11.3	0.6	−28.1	1.60	0.058	6	11.7	0.8	15.9	1.2
14	11.3	0.6	−34.1	2.00	0.046	6	16.5	1.9	22.4	2.7
15	11.3	0.6	−40.6	2.40	0.038	6	13.1	2.7	17.8	3.6
16	11.3	0.6	−48.0	2.80	0.033	6	10.0	1.0	13.6	1.3
R5-0.75X-G3										
17	5.4	0.75	−3.6	0.36	0.300	6	92.6	0.4	100.0	0.4
18	5.4	0.75	−7.6	0.72	0.150	6	84.5	1.9	91.3	2.1
19	5.4	0.75	−10.7	1.00	0.109	6	44.9	1.4	48.5	1.5
20	5.4	0.75	−15.2	1.45	0.075	6	13.0	1.6	14.1	1.8
21	5.4	0.75	−16.8	1.60	0.068	6	13.5	0.6	14.5	0.6
22	5.4	0.75	−21.5	2.00	0.054	6	7.8	0.5	8.5	0.5
23	5.4	0.75	−26.0	2.40	0.045	6	6.0	0.5	6.4	0.6
24	5.4	0.75	−29.8	2.80	0.039	6	6.0	1.0	6.5	1.1
R11-0.75X-G7										
25	11.3	0.75	−4.0	0.24	0.450	6	87.6	0.2	100.0	0.3
26	11.3	0.75	−6.1	0.36	0.300	6	87.4	1.4	99.8	1.6
27	11.3	0.75	−12.6	0.72	0.150	6	75.4	1.3	86.1	1.4
28	11.3	0.75	−17.8	1.00	0.107	6	41.1	1.5	46.9	1.7
29	11.3	0.75	−25.3	1.43	0.075	3	16.8	0.3	19.2	0.3
30	11.3	0.75	−28.1	1.60	0.067	6	12.3	0.4	14.0	0.4
31	11.3	0.75	−34.1	2.00	0.054	6	9.3	0.8	10.6	0.8
32	11.3	0.75	−40.6	2.40	0.045	6	9.9	2.8	11.3	3.2
33	11.3	0.75	−48.0	2.80	0.038	6	5.6	1.1	6.4	1.3
R5-1X-G4										
34	5.4	1.0	−5.1	0.49	0.300	9	93.7	1.2	100.0	1.2
35	5.4	1.0	−10.4	0.98	0.150	12	83.6	1.5	89.2	1.6
36	5.4	1.0	−10.7	1.00	0.150	9	81.1	0.7	86.5	0.7
37	5.4	1.0	−16.8	1.60	0.092	9	39.3	4.5	42.0	4.8
38	5.4	1.0	−20.9	1.96	0.075	12	18.2	1.3	19.4	1.4
39	5.4	1.0	−21.5	2.00	0.073	9	28.7	3.3	30.6	3.6
40	5.4	1.0	−26.0	2.40	0.061	12	9.7	2.1	10.3	2.3
41	5.4	1.0	−29.8	2.80	0.052	9	8.8	2.5	9.4	2.6

TABLE IIA *continued*

Condition	R	Relative NaCl tonicity	Temperature	Molal. NaCl	Fraction unfrozen	*N*	Absol. survl.		Norm. survl.	
							Mean	SE	Mean	SE
R11-1X-G9										
42	11.3	1.0	−5.6	0.33	0.450	9	96.1	0.9	100.0	1.0
43	11.3	1.0	−8.6	0.49	0.300	9	96.2	0.8	100.1	0.8
44	11.3	1.0	−17.7	0.99	0.150	12	85.6	0.9	89.1	0.9
45	11.3	1.0	−28.1	1.60	0.092	6	49.7	1.6	51.7	1.7
46	11.3	1.0	−33.8	1.96	0.075	6	23.8	1.1	24.8	1.1
47	11.3	1.0	−34.1	2.00	0.073	9	19.9	2.3	20.7	2.4
48	11.3	1.0	−40.6	2.40	0.061	6	16.0	0.8	16.7	0.8
49	11.3	1.0	−48.0	2.80	0.052	9	12.9	1.2	13.4	1.3

*See Table I for solution description.

TABLE IIB

SURVIVALS OF HUMAN ERYTHROCYTES SUSPENDED IN VARIOUS GLYCEROL-NaCl-WATER SOLUTIONS, FROZEN AT 1.8°C/MIN TO VARIOUS SUBZERO TEMPERATURES, AND THAWED RAPIDLY

Condition	R	Relative NaCl tonicity	Temperature	Molal. NaCl	Fraction unfrozen	*N*	Absol. survl.		Norm. survl.	
							Mean	SE	Mean	SE
			(°C)	(m_s)	(U)		(%)		(%)	
R5-2X-G9										
50	5.4	2.0	−10.9	1.02	0.304	15	92.6	1.9	100.0	2.0
51	5.4	2.0	−16.8	1.60	0.190	6	79.6	0.9	86.0	0.9
52	5.4	2.0	−21.5	2.00	0.160	9	61.4	1.5	66.3	1.6
53	5.4	2.0	−22.2	2.07	0.150	9	53.2	5.3	57.4	5.7
54	5.4	2.0	−26.0	2.40	0.130	9	42.2	3.3	45.5	3.6
55	5.4	2.0	−29.8	2.80	0.110	9	29.1	1.9	31.5	2.1
R11-2X-G18										
56	11.3	2.0	−12.9	0.73	0.450	9	91.9	1.0	100.0	1.1
57	11.3	2.0	−17.8	1.00	0.330	6	90.2	0.7	98.1	0.8
58	11.3	2.0	−19.6	1.09	0.300	9	91.2	1.1	99.2	1.2
59	11.3	2.0	−28.1	1.60	0.210	6	66.8	3.7	72.7	4.0
60	11.3	2.0	−34.1	2.00	0.160	9	37.9	2.8	41.3	3.0
61	11.3	2.0	−37.1	2.20	0.150	6	30.1	2.0	32.7	2.1
62	11.3	2.0	−40.6	2.40	0.140	9	28.8	1.8	31.3	2.0
63	11.3	2.0	−48.0	2.80	0.120	9	26.2	0.7	28.5	0.7
R5-3X-G13										
64	5.4	3.0	−10.7	1.00	0.490	6	87.1	2.8	100.0	3.3
65	5.4	3.0	−16.8	1.60	0.310	6	83.7	0.7	96.1	0.8
66	5.4	3.0	−17.6	1.65	0.300	9	84.6	1.5	97.1	1.8
67	5.4	3.0	−21.5	2.00	0.250	9	68.9	0.7	79.1	0.8
68	5.4	3.0	−26.0	2.40	0.210	9	42.4	3.0	48.7	3.5
69	5.4	3.0	−29.8	2.80	0.180	9	24.2	1.9	27.7	2.1
70	5.4	3.0	−34.4	3.30	0.150	9	11.7	3.2	13.4	3.7

TABLE IIB *continued*

Condition	R	Relative NaCl tonicity	Temperature	Molal. NaCl	Fraction unfrozen	N	Absol. survl.		Norm. survl.	
							Mean	SE	Mean	SE
			(°C)	(m_s)	(U)		(%)		(%)	
R11-3X-G26										
71	11.3	3.0	−17.8	1.00	0.560	6	91.6	0.6	100.0	0.7
72	11.3	3.0	−22.1	1.24	0.450	9	90.7	0.6	99.1	0.6
73	11.3	3.0	−28.1	1.60	0.350	6	87.1	1.0	95.1	1.1
74	11.3	3.0	−32.2	1.86	0.300	9	72.4	3.0	79.1	3.3
75	11.3	3.0	−34.1	2.00	0.280	9	67.0	3.3	73.2	3.5
76	11.3	3.0	−40.6	2.40	0.230	9	57.8	5.0	63.1	5.5
77	11.3	3.0	−48.0	2.80	0.200	9	47.4	3.9	51.8	4.2
R5-4X-G18										
78	5.4	4.0	−10.7	1.00	0.700	6	90.7	1.3	100.0	1.5
79	5.4	4.0	−16.8	1.60	0.440	6	86.6	1.6	95.5	1.8
80	5.4	4.0	−21.5	2.00	0.350	9	75.0	1.6	82.7	1.7
81	5.4	4.0	−25.4	2.34	0.300	6	61.2	1.2	67.5	1.3
82	5.4	4.0	−26.0	2.40	0.290	9	56.9	1.8	62.8	2.0
83	5.4	4.0	−29.8	2.80	0.250	9	43.7	1.7	48.2	1.9
R11-4X-G35										
84	11.3	4.0	−17.8	1.00	0.860	6	84.1	1.4	100.0	1.6
85	11.3	4.0	−28.1	1.60	0.540	6	81.6	0.9	97.1	1.1
86	11.3	4.0	−32.9	1.91	0.450	9	80.2	1.7	95.4	2.0
87	11.3	4.0	−34.1	2.00	0.430	9	74.5	2.4	88.6	2.9
88	11.3	4.0	−40.6	2.40	0.360	12	69.1	1.7	82.2	2.0
89	11.3	4.0	−48.0	2.80	0.310	9	58.5	4.7	69.5	5.6
90	11.3	4.0	−49.2	2.86	0.300	6	63.3	1.1	75.3	1.3

survival" gives the actual percentages of unhemolyzed cells. In most cases, the absolute survivals were well above 90% at the highest freezing temperature in each solution set. However, they were 74% in the 0.6 times isotonic solutions (conditions 1 and 9).

As shown in Table III, the hemolysis produced at the highest temperature for each solution was not a result of ice formation, since comparable hemolysis occurred in samples supercooled to about the same temperatures. Because we were interested in the influence of freezing on hemolysis, we decided that it could be best assessed by normalizing the absolute survivals for a given solution to the survival at the highest temperature used for that solution. The normalized survivals are given in the two right-most columns of Table II.

Three conclusions emerge from the results: one is that the survivals drop with decreasing temperature. The second is that the higher the starting concentration of glycerol plus NaCl in a solution of given R, the lower the temperature at which the drop occurs. For example, in condition 19, 50% survival occurred at ~ − 10.7°C, whereas in condition 68 it occurred at ~ −26°C. The initial solution in the former case (condition 19) was 0.4 molal glycerol in 0.11 molal NaCl (Table I); the initial solution in the latter case (condition 68) was 1.7 molal glycerol in 0.5 molal NaCl. The third conclusion is that the drop in survival occurs at a lower temperature in the R = 11.3 solutions than in the R = 5.4 solutions (e.g., compare conditions 19 and 28). These relations are more self-evident when the data are plotted. To economize on

TABLE III

SURVIVAL OF HUMAN ERYTHROCYTES IN SUPERCOOLED GLYCEROL-NaCl-WATER SOLUTIONS VS. SURVIVAL IN SOLUTIONS FROZEN TO COMPARABLE TEMPERATURES

Solution*	First frozen sample in set			Unseeded control	
	Condition‡	Temperature	Survival§	Temperature	Survival
		(°C)	(%)	(°C)	(%)
R5-0.6X-G3	1	−3.1	83.9	−2.8	95.1
R5-0.75X-G3	17	−3.6	92.6	−3.3	98.0
R5-1X-G4	34	−5.1	96.3	−5.1	94.7
R5-2X-G9	50	−10.8	99.0	−6.5	98.7
R5-3X-G13	64	−10.7	93.5	−8.7	93.5
R5-4X-G18	78	−10.7	92.1	−10.3	93.0
R11-0.6X-G6	9	−5.2	73.6	−5.0	79.9
R11-0.75X-G7	25	−4.0	87.6	−3.7	90.8
R11-1X-G9	42	−5.6	94.3	−5.6	94.3
R11-2X-G18	56	−12.9	90.2	−12.0	90.5
R11-3X-G26	71	−17.8	91.6	−15.7	91.1
R11-4X-G35	84	−17.8	84.0	−17.6	79.6
	Mean survival		89.9 ± 2.0		91.6 ± 1.8

*See Table I.

‡See Table II, column 1.

§The survival values in Table II are the means of 6–15 replicate samples from 2–5 experiments. The survival values here are only from those experiments in which there were supercooled controls.

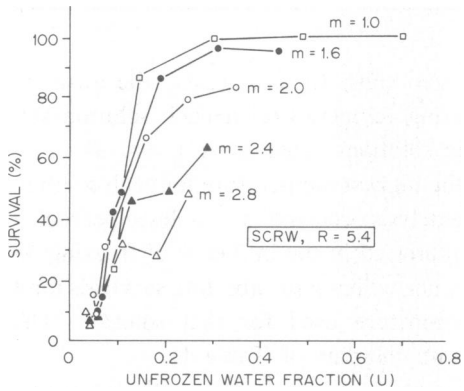

FIGURE 7

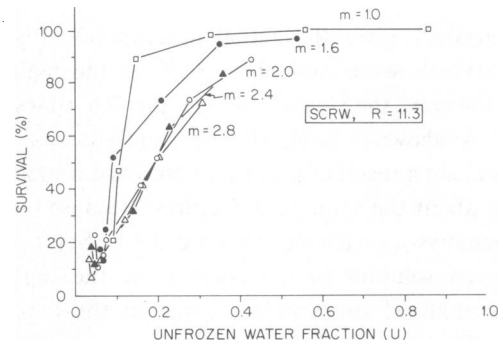

FIGURE 8

FIGURE 7 Normalized survivals of human erythrocytes as a function of U for cells in glycerol-NaCl-water frozen slowly at 1.8°C/min to various subzero temperatures and warmed rapidly (SCRW). R = 5.4. U was varied independently of m_s by appropriate choice of initial salt tonicity and the minimum freezing temperature. The data points forming the individual curves are from the following data in Table II: m_s = 1.0 (condition nos. 3, 19, 35, 36, 50, 64, 78); m_s = 1.6 (conditions 5, 21, 37, 51, 65, 79); m_s = 2.0 (conditions 6, 22, 39, 52, 53, 67, 80); m = 2.4 (conditions 7, 23, 40, 54, 68, 81, 82); m_s = 2.8 (conditions 8, 24, 41, 55, 69, 83).

FIGURE 8 Normalized survivals of human erythrocytes as a function of the U in glycerol-NaCl-water solutions. Same as Fig. 7 except R = 11.3. The data points forming the individual curves are from the following data in Table II: m_s = 1.0 (condition 11, 28, 44, 57, 71, 84); m_s = 1.6 (condition 13, 30, 45, 59, 73, 85); m_s = 2.0 (condition 14, 31, 46, 47, 60, 75, 87); m_s = 2.4 (condition 15, 32, 48, 62, 76, 88); m_s = 2.8 (condition 16, 33, 49, 63, 77, 89, 90).

space, we have not included such plots. We can, however, provide them to interested readers.

Survival vs. U at constant m_s. The data in Table II were sorted by computer in order of increasing m_s, and within m_s in order of increasing U. We then extracted those data from the sorted table for which there were six or more values of U for a given m_s, and plotted the normalized survivals vs. U in Figs. 7 (R = 5.4) and 8 (R = 11.3). (Copies of the sorted table will be furnished on request.) For example, the curve in Fig. 7 labeled m = 1.0 includes conditions 3, 19, 35, 36, 50, 64, and 78 from Table II, the sets for which m_s lay between 0.98

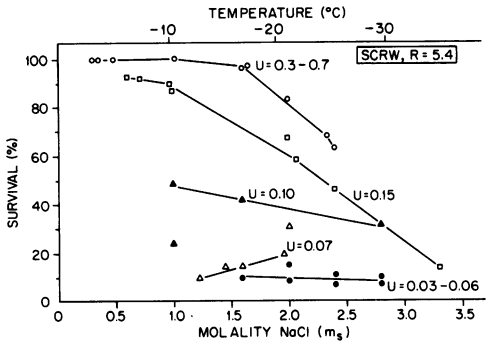

FIGURE 9

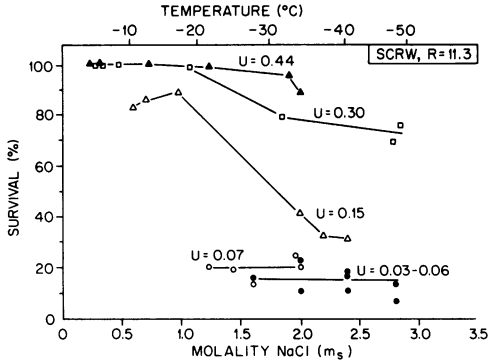

FIGURE 10

FIGURE 9 Normalized survivals of human erythrocytes as a function of m_s in the unfrozen portions of glycerol-NaCl-water solutions when R = 5.4. m_s was varied independently of U by appropriately varying the initial NaCl tonicity and glycerol concentrations. The cooling rate was 1.8°C/min. The top abscissa shows the temperatures corresponding to the various values of m_s. The data points forming the individual curves are from the following data in Table II:

U		
Indicated	Actual	Condition No.
0.03–0.06	0.033–0.061	5, 6, 7, 8, 22, 23, 24, 40, 41
0.07	0.068–0.075	4, 20, 21, 38, 39
0.10	0.092–0.110	3, 19, 37, 55
0.15	0.130–0.160	2, 18, 35, 36, 52, 53, 54, 70
0.3–0.7	0.290–0.700	1, 17, 34, 50, 64, 65, 66, 78, 79, 80, 81, 82

FIGURE 10 Normalized survivals of human erythrocytes as a function of m_s in unfrozen portions of glycerol-NaCl-water when R = 11.3. The top abscissa shows the temperatures corresponding to the various values of m_s. The data points forming the individual curves are from the following data in Table II:

U		
Indicated	Actual	Condition No.
0.03–0.06	0.033–0.061	13, 14, 15, 16, 31, 32, 33, 48, 49
0.07	0.067–0.075	12, 29, 30, 46, 47
0.15	0.14–0.16	10, 27, 44, 60, 61, 62
0.30	0.30–0.31	9, 26, 43, 58, 74, 89, 90
0.44	0.43–0.45	25, 42, 56, 72, 86, 87

and 1.02. The data sets used to construct the other curves are given in the figure legends. The results for both R values are closely comparable and may be summarized as follows.

(*a*) Survival dropped below 30% whenever the fraction of unfrozen water was 0.10 or below, regardless of the m_s.

(*b*) As U was increased from 0.05 to above 0.10, the percent survival rose sharply, regardless of m_s.

(*c*) The salt concentration, however, determined the maximum survival at high values of U. The higher m_s, the lower the maximum.

Survival vs. m_s at constant U. The data in Table II were then re-sorted in order of increasing U, and within U in order of increasing m_s. (Copies available on request.) We then extracted data sets from the re-sorted table for five different ranges of U values, and for each range we made the plots of normalized survival vs. m_s shown in Figs. 9 (R = 5.4) and 10 (R = 11.3). For example, the curve labeled U = 0.03–0.06 in Fig. 10 includes conditions 13–16, 31–33, 48, and 49 from Table II.

The results fall into three broad categories.

(*a*) When U was 0.03–0.07, survival was 20% or less over salt concentrations ranging from 1.2 to 2.8 molal.

(*b*) When U was 0.3 or above, survival was 65% or higher over m_s ranging from 0.3 to 2.8 molal.

(*c*) Values of U between 0.16 and 0.09 represented a transition region from high survival to low, and in this transition region survivals tended to decrease with increasing m_s.

COOLING AT 0.6°C/MIN, RAPID THAW Freezing at 1.8°C/min is slow enough to allow red cells to remain in osmotic equilibrium with the external solution, and presumably it is slow enough to produce phase compositions in the external solution that come close to matching those described by the curves in Figs. 1, 2, and 6. To check on the validity of this presumption, we repeated the experiments with the R = 5.4 solutions using a cooling rate some threefold slower. Fig. 11 shows absolute percent survival vs. temperature for cells cooled at 0.6°C/min in five R = 5.4 solutions. Fig. 12 shows the normalized percent surivival vs. U for a series of NaCl concentrations, and Fig. 13 shows the normalized survival vs. m_s for three ranges of U. We can supply the detailed tabular data analogous to Table II and its re-sorted versions upon request.

The results are quite comparable to those obtained with the threefold higher cooling rates, namely:

(*a*) When cells were frozen to temperatures that reduced U to 0.04–0.075, only ~15% survived irrespective of m_s, which varied from 1.4 to 2.8 molal (Figs. 12 and 13).

(*b*) Survival rose dramatically to 75% or higher when U was 0.15 or higher. This held for m_s ranging from 0.4 to 2.4 molal.

(*c*) When U was 0.15 or above, a detrimental effect of NaCl concentration was noticed only when m_s was 2.4–3.4 molal. Even here, in the worst case, about half of the cells survived.

DISCUSSION

Data such as those in Fig. 3 are the basis for the widely held view that injury to slowly frozen human erythrocytes (and other cells) results from the high concentrations of electrolytes that are produced in and around cells as water is removed from the solution in the form of ice. That

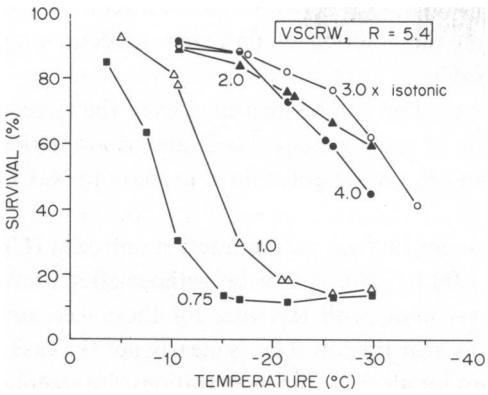

FIGURE 11

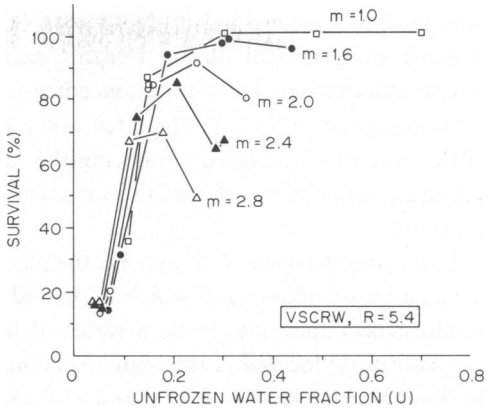

FIGURE 12

FIGURE 11 Absolute survivals of human erythrocytes after cells in various concentrations of glycerol and NaCl were cooled very slowly (0.6°C/min) to various subzero temperatures, then warmed rapidly (VSCRW).

FIGURE 12 Normalized survivals of very slowly frozen human erythrocytes as a function of U. Like Fig. 7 except that the cooling rate was 0.6°C/min instead of 1.8°C/min.

figure shows that survival drops abruptly as the concentration of NaCl in the unfrozen channels rises above about 1 molal. The data in Fig. 3 pertain to cells suspended in solutions of glycerol-isotonic NaCl-water. They compare closely with our previous findings for cells in glycerol-isotonic buffered saline-water, where the NaCl was buffered to pH 7.0 with 0.01 M phosphate buffer (Souzu and Mazur, 1978). We used unbuffered NaCl as the salt in the present case because the most complete published phase diagram data have been obtained with it as the sole salt.

The reason the NaCl (and glycerol) concentrate during freezing is that the precipitation of pure ice progressively restricts the solutes to smaller and smaller volumes of unfrozen liquid.

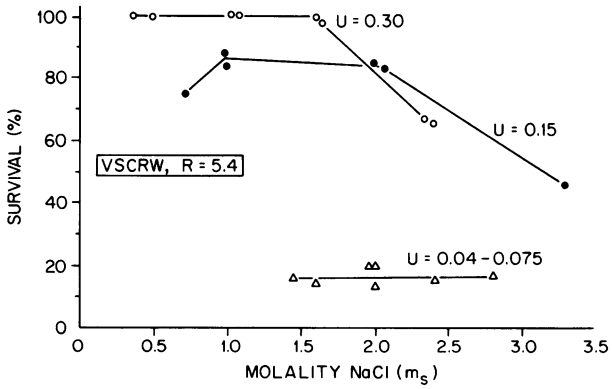

FIGURE 13 Normalized survivals of very slowly frozen erythrocytes as a function of m_s in the unfrozen portion of glycerol-NaCl-water solutions. Like Fig. 9 except that the cooling rate was 0.6°C/min rather than 1.8°C/min.

And indeed, as shown in Fig. 5, the survival data in Fig. 3 can equally well be interpreted to indicate that survival during freezing depends on the fraction of unfrozen water at any temperature rather than on the concentration of NaCl.

As discussed in Results, the effects of salt concentration can be separated from the effects of the unfrozen fraction by removing the restriction of using isotonic NaCl and allowing the starting concentration of NaCl to vary while holding R, the weight ratio of glycerol to NaCl, constant.

First, consider Figs. 7, 8, and 12, the plots of percent survival vs. the fraction unfrozen (U) for a series of solutions in which m_s varied from 1.00 to 2.80. If U were without effect, one would expect the plots to be a series of horizontal lines, with the lines for the lower salt concentrations located in the higher survival regions near the top. This is clearly not the case. In the region between $U = 0.03$ and 0.12, the curves for all the salt concentrations rise steeply and are nearly superimposable. This is to say, survival is almost totally dependent on U in the range where > 88% of the water is frozen. It is only when < 80–85% of the water is frozen that we see signs of a series of parallel horizontal lines indicating a controlling influence of m_s.

Second, if salt concentration rather than the fraction of frozen water were the major factor in injury, then plots of survival vs. m_s ought to look like the data in Fig. 3 regardless of the value of U. That is, all the survival curves should drop sharply with increasing m_s. But when the contributions of m_s and U are separated out (Figs. 9, 10, and 13), we see that this is not the case. When U is < 8%, the survival curves are low and nearly horizontal over a broad range of m_s (1.2 to 2.8 molal). Conversely, when U is ≥ 30%, survivals are high over a broad range of m_s. The curves only drift downward gradually when m_s attains very high values.

The conclusion seems unavoidable that the primary factor responsible for erythrocyte hemolysis is the fraction of unfrozen water, and that salt concentration plays only a secondary role. Before eliminating the qualifier "seems," however, it is necessary to examine the role, if any, of two potentially confounding factors, namely, temperature and glycerol concentration.

Glycerol-NaCl-water solutions with R values of 5.4 and 11.3 attain identical values of m_s and U at different temperatures. This permits an assessment of the role of temperature. The assessment was made by comparing the survivals after treatments that yielded the same values of U and m_s and differed only in the values of R and temperature; i.e., compare conditions 8 and 16, 7 and 15, 33 and 24, 23 and 32, 14 and 6, 41 and 49, etc. in Table II. In all there are 23 R pairs between $U = 0.033$ and 0.150. (These pairs can be identified more readily in the re-sorted version of Table II referred to above. At $U > 0.150$, survivals are high and insensitive to other conditions.) Within these 23 pairs, there was no mean difference between the percentage survivals in the $R = 11.3$ solutions and in the $R = 5.4$ solutions, in spite of the fact that the $R = 11.3$ solution in each pair was cooled an average of 12.2 ± 0.9°C lower than its $R = 5.4$ counterpart. (To be exact, the mean difference in absolute survivals between $R = 5.4$ and $R = 11.3$ pairs was +0.2 ± 1.7%, and the mean difference in normalized survivals was −0.3 ± 1.8%.)

The fact that the survivals are the same in $R = 5.4$ and $R = 11.3$ solutions that have attained the same values of m_s and U leads to a second important conclusion; namely, that survival was not affected by the concentration of glycerol. The concentration of glycerol in the unfrozen portions of each of the 23 pairs just discussed was about twice as high in the $R = 11.3$ solution as in its $R = 5.4$ counterpart.

We must conclude, therefore, that temperature and glycerol concentration have no observable effect on survival other than their role in affecting the values of U and m_s.

Effect of Method of Expressing NaCl Concentration and Fraction Unfrozen

We have expressed both the concentrations of NaCl in the unfrozen channels and the fractions unfrozen in terms of liquid water. The molal units used for the former seem appropriate to us because they are more directly related to the important underlying factors such as osmotic pressure and water activity than are concentration units like weight fraction or molarity. The experimental results are such, however, that the method of expressing salt concentration and fraction unfrozen will not change the conclusion that survival is primarily dependent on the latter and only secondarily on the former. If, for example, the abscissas of Figs. 9, 10, and 13 were expressed as percent by weight of NaCl instead of molality of NaCl, the weight percentages would not be spaced equally; but that would not change the conclusions drawn from the figure. Similarly, if the data in these three figures were plotted as groups of values for the fraction of the solution that remains unfrozen (L) rather than in terms of the fraction of unfrozen water (U), the numerical limits of the groups would change, but the conclusions would not. For example, in Fig. 10, the ranges of L that correspond to the five ranges of U are 0.09–0.15, 0.13–0.16, 0.20–0.32, 0.34–0.57, and 0.49–0.66. The equations given in the beginning of Results and the equations and data in Rall et al. (1978) will permit any interested reader to make the necessary quantitative conversion from NaCl molality to weight percent NaCl and from weight fraction of unfrozen water to weight fraction of unfrozen solution. Fahy (1980) has recently published a somewhat different approach to constructing the desired isopleths for glycerol-NaCl-water solutions, and a different set of equations for converting from one set of concentration units to another. His and our approaches yield similar estimates (±2%) for the subzero temperatures at which various concentrations of NaCl are attained.

In this discussion we consider the composition of the unfrozen portions of the glycerol-NaCl solutions at any given bath temperature to be the equilibrium composition described by the phase diagram. Two lines of evidence support this view. One is that the temperature of the contents of the samples agreed within 0.2°C (the limit of resolution of the recorder) with the more precisely determined bath temperature. Seeding ensured that ice was present in all the samples and that extensive supercooling did not occur. The phase law states that if we specify the temperature of a given ice-aqueous solution system (and the pressure is constant), the composition of the aqueous solution is fixed. Hence, if the temperature of the samples was close to that specified, so too must have been their phase composition. However, since sample temperature was measured with only a single thermocouple, it is possible that thermal gradients were present within the tubes. If so, the gradients should have been appreciably less in the experiments involving very slow cooling (0.6°C/min). But the survivals obtained with very slow cooling as a function of m_s and U were quantitatively similar to those obtained with slow cooling at 1.8°C/min. Indeed, where differences were present, the former was even less damaging than the latter, a finding that is opposite from what one would expect were the volume and composition of the unfrozen fraction to be lagging the equilibrium values at the higher cooling rate.

Physical Significance of U

The phase diagram data provide macroscopic information on the fraction of unfrozen water in the system, but they provide no information on the microscopic arrangement of unfrozen channels, and none on the distribution of cells within the channels. A number of microscopic studies, however, have demonstrated that the unfrozen portions of partly frozen glycerol-NaCl solutions consist of irregular channels between the ice crystals, and that at low cooling rates the erythrocytes are located within these channels (e.g., Rapatz et al., 1966). The microscopic studies further indicate that channel diameters shrink with decreasing temperature. Inasmuch as decreasing temperatures produce decreasing values of the macroscopic unfrozen water fraction U, it seems reasonable to assume that the cell damage associated with low values of U means on the microscopic scale that the damage is associated with a reduction in the diameter of the channels or with some other alteration of their geometry.

State of the Erythrocytes in the Channels

Although our experiments bear directly only on the relation between erythrocyte survival and the physical state of the *extracellular* solution, we can draw some inferences about the relation between survival and the physical state of the cells themselves.

Theoretical studies by Mazur (1963) and more recent theoretical and experimental studies by Silvares et al. (1975), and Diller (1979) have shown that human erythrocytes cooled at 100°C/min or less remain close to osmotic equilibrium with the partially frozen medium that surrounds them. And we cooled the cells at 1.8°C/min or less.

Not only were the cells in osmotic equilibrium with the surrounding solution in the unfrozen channels throughout cooling, they were also in equilibrium with respect to the intra- and extracellular concentrations of glycerol. The human erythrocyte is very permeable to glycerol but almost impermeable to Na^+. The 30–40-min contact with glycerol at room temperature before freezing was several-fold longer than that required to achieve equilibration with respect to glycerol (Mazur and Miller, 1976). Consequently, the intra- and extracellular concentrations of glycerol were equal before freezing.

Although cell volume before freezing is determined chiefly by the concentration of the impermeant species in the medium (here NaCl), there is also a small contribution from the volume occupied by the intracellular glycerol. In general the volume of a cell (V_c) relative to its volume in isotonic NaCl is (Mazur and Miller, 1976):

$$V_c = (V + d + n_g\bar{v}_g/V_{iso})/(1 + d), \tag{5}$$

where V is the volume of cell water relative to the volume of water in the isotonic cell, d is the volume ratio of indigenous cell solids to cell water in the isotonic cell, n_g is the number of moles of permeating solute (here, glycerol), $\bar{v}_g$ is the partial molal volume of glycerol, and V_{iso} is the absolute volume (cm^3) of intracellular water in the isotonic cell. But $n_g/V_{iso} = m_g^o/1{,}000$, where m_g^o is the molality of glycerol in the unfrozen medium. To a close approximation for the salt concentrations used here, $V = m_{iso}^o/m_s^o$, where m_{iso}^o and m_s^o are the isotonic and nonisotonic molalities of NaCl. (To be exact the equation requires the ratio of the osmolalities.) Accordingly, Eq. 5 becomes

$$V_c = (m_{iso}^o/m_s^o + d + m_g^o\bar{v}_g/1{,}000)/(1 + d). \tag{6}$$

The partial molal volume of glycerol ($\bar{v}_g$) is 71 cm^3/mol, d for the human erythrocyte is 0.4 (Mazur and Miller, 1976), and we have defined isotonic NaCl as 0.147 molal. Hence, here $V_c = (0.147/m_s^\circ + 0.4 + 7\ 1m_g^\circ/1{,}000)/1.4$.

Table I gives the values of m_s° and m_g° for the 12 solutions that we used, and the corresponding values of V_c computed by this equation. Thus, before freezing, the cells in the 0.6 and 0.75 times isotonic solutions were swollen above isotonic volume and the cells in 2.0, 3.0 and 4.0 times isotonic solutions were shrunken below isotonic volume.

As freezing progresses, the rise in the concentration of extracellular glycerol and NaCl causes an increase in extracellular osmotic pressure. The fact that the cells remained in osmotic equilibrium means that their osmotic pressure increased at an equivalent rate. Since the human erythrocyte is 3,000 times more permeable to water than to glycerol (Mazur and Miller, 1976), the increase in intracellular osmotic pressure must result almost entirely from the efflux of intracellular water, i.e., the cells shrink.

The osmotic pressure of the solution in the channels is completely defined by its values of R and temperature, and it is independent of U and presumably of the manner in which the unfrozen solution is distributed in microscopic channels. If the cells maintain osmotic equilibrium with the external solution by decreasing their volume during freezing, we must conclude that both their volume and the concentration of solutes within them are also dependent only on the values of R and temperature and are independent of U.

But as we have seen, the *survival* of the erythrocytes shows almost the opposite dependence: survival is dependent chiefly on U, the fraction of unfrozen water, and is only secondarily dependent on m_s, the solute concentration. Since survival depends chiefly on U, but cell volume and the concentration of intracellular solutes are nearly independent of U, it is difficult to avoid the conclusion that cell shrinkage and the concentration of extra- and intracellular solutes have little to do with erythrocyte injury during freezing and thawing. Rather, cell survival appears to depend chiefly on the volume or configuration of the aqueous channel in which the cell finds itself.

This is a surprising result. It means that, contrary to the current consensus, slow freezing injury in at least this instance is only secondarily a manifestation of the altered solution composition during freezing (solution effects). It appears to be more a manifestation of physical consequences of the shrinkage of the aqueous channels to below rather discrete values.

Possible Explanations of the Dependence of Survival on the Unfrozen Fraction

Why should the survival of the red cell depend more on the amount of unfrozen solution in the sample than on the composition of that solution? The following facts may be relevant to the answer.

(*a*) Bovine erythrocytes in 3 M glycerol in isotonic saline at 0°C hemolyze extensively when syringed through a hypodermic needle. They do not show this sensitivity at room temperature (Mazur et al., 1974).

(*b*) Bovine erythrocytes undergo extensive hemolysis when forced to undergo rapid osmotic changes in volume in unfrozen multimolar solutions of glycerol-NaCl-water at -7 to $+0$°C. Comparable volume changes at temperatures of 10–20°C are not detrimental (Leibo, 1976).

(*c*) The osmotic shrinkage of mouse embryos in unfrozen hyperosmotic glycerol-NaCl-

water solutions occurs isotropically with no deformation of the zona pellucida (Jackowski et al., 1980). But the analagous osmotic shrinkage that occurs when solutions become hyperosmotic during freezing occurs anisotropically; the embryo itself and the zona pellucida become highly distorted (Leibo et al., 1978).

These facts suggest two things to us: (*a*) The surfaces of at least some cells may become "brittle" at temperatures of 0°C and below. (*b*) Cells in the unfrozen channels between ice crystals are subjected to forces in addition to those like osmotic pressure that are due to changes in the composition of the unfrozen solution. One possible force is shear. A second is the following: Although the total volume of unfrozen solution at any temperature is determined by phase-rule thermodynamics, restrictions on the lattice structure and morphology of the ice crystals put constraints on the geometries of aqueous channels. For example, the channels formed in rapidly frozen solutions in which the crystals are highly dendritic are very different from those formed between the blunt plate-like ice crystals that develop during slow freezing (Rapatz et al., 1966; Luyet and Rapatz, 1969). The restrictions on the geometries of the channels could force the cells in those channels to become deformed. And serious deformation at subzero temperatures could be damaging.

The decrease in the volume of unfrozen channels during freezing not only increases the chance of interactions between cells and the ice crystals that border the channels, it also increases the chances of cell-to-cell interactions. Indeed, Pegg (1981) has recently reported that the hemolysis of human erythrocytes that accompanies a given slow freezing treatment is increased greatly when the erythrocytes are frozen at a hematocrit above 60%, and Nei (1968) has found that it is decreased substantially when cells are frozen at a hematocrit of only 4%. Although this cell-crowding effect could well have a similar genesis to the effects reported here, cell-cell interactions are unlikely to explain our results, since the hematocrit of our cell suspensions was only 2%. We will report on the effects of altering the hematocrit in a subsequent publication.

We will also report on the effect of warming rate, a potentially important matter since slowly frozen human erythrocytes have been shown to be more injured by rapid thawing at the rate used in the present study than by slow thawing (Miller and Mazur, 1976). However, it will be seen that the use of slow thawing does not alter the conclusions reached here.

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